REMARKS

Before this Amendment, claims 12-24 were pending. By this Amendment, claims 12 and 20-24 have been canceled without prejudice to the Applicants' right to pursue those claims in continuing applications. Accordingly, claims 13-19 are now pending.

Claims 13-15 have been amended to place those claims into independent form. Claims 16-19 have been amended so as to depend only from claims 13-15.

Continuity data

The specification has been amended to recite continuity data, as requested in the Office Action.

The rejection under 35 U.S.C. §112, first paragraph

Claims 13-20 were rejected for lack of enablement.

The Office Action reviewed some of the factors involved in an enablement analysis that are discussed in *In re Wands*, 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). According to the Office Action, these factors lead to a conclusion of non-enablement.

The Applicants respectfully disagree with the Office Action's review of certain of the *Wands* factors. It is the Applicants' position that a more accurate consideration of those *Wands* factors would be as follows:

State of, or the amount of knowledge in, the prior art

The Office Action, at page 4, stated that "apoptosis is a complex series of cellular events." Although not explicitly stated, presumably the Office Action's position is that this complexity favors a conclusion that the present claims lack enablement.

The Applicants do not contest that apoptosis is complex. However, this complexity does not favor a conclusion of lack of enablement because it is not necessary to understand the complexity of apoptosis in order to practice the claimed invention. It is merely necessary to understand how to administer xenon to organs that are to be transplanted or to patients after eye laser surgery or patients suffering from sepsis. Since xenon is a substance that is well known to have medical uses, methods of administering xenon are well known. Those skilled in the art would know how to adapt those methods without undue experimentation in order to administer xenon for the purposes recited in the present claims.

Level or degree of predictability, or lack thereof, in the prior art

The Office Action, at page 4, stated that "much is left unknown" about apoptosis.

As for the complexity of apoptosis, the Applicants do not disagree with this, but wish to point out that, like the complexity of apoptosis, the fact that much in still unknown about apoptosis does not weigh in favor of a conclusion of lack of enablement for the present claims.

Again, these is no need for those skilled in the art to understand all that is still unknown about apoptosis in order administer xenon for the purposes recited in the present claims.

Amount of guidance or direction provided by the inventor

The Office Action, at page 5, stated: "[T]here is a lack of guidance in the specification as to how one would extrapolate the cell based neuroprotection experiments to cellular damage, apoptotic cell death after laser eye surgery or protecting against sepsis."

The Applicants do not agree that there is a lack of guidance in the specification. The experiments described in the specification provide sufficient guidance because:

- The experiments were conducted in a well known model system for apoptosis.
- The experiments]demonstrate that xenon almost completely inhibits the activity of caspases 3 and 7
- Caspases 3 and 7 have a central role in apoptosis.
- The effects of xenon were demonstrated in two very different cell types.
- Xenon completely protected against apoptosis.
- The cell death that occurs during the circumstances recited in the claims (organ transplantation, eye laser surgery, and sepsis) is known to be largely due to apoptosis.

Staurosporine treatment of tissue culture cells is a well-recognized model system for apoptosis

As disclosed in the specification, at page 11, staurosporine is "generally considered a model apoptosis inducer."

(B) Induction of apoptosis

Apoptosis was induced using staurosporine. Staurosporine is an antibiotic originally discovered by Omura et al., J. Antibiot. 30 (1977), 275. It is generally considered a model apoptosis inducer when present in micromolar concentration (Tamaoki et al., BBRC 135 (1986), 397; Nakano et al., J. Antibiot. 40 (1987), 706; Ruegg and Burgess, TIPS 10 (1989), 218; Bertrand et al., Exp. Cell Res. 211 (1994), 314; Wiesner and Dawson, CLAO J. 24 (1996), 1418; Boix et al., Neuropharmacology 36 (1997), 811; Kirsch et al., J. Biol. Chem. 274 (1999), 21155; Chae et al., Pharmacol. Res. 42 (2000), 373; Heerdt et al., Cancer Res. 60 (2000), 6704; Bijur et al., J. Biol. Chem. 275 (2000), 7583; Scarlett et al., FEBS Lett. 475 (2000), 267; Tainton et al., BBRC 276 (2000), 231; Tang et al., J. Biol. Chem. 275 (2000), 9303; Hill et al., J. Biol. Chem. 276 (2001), 25643). Cells were seeded in 24-well plates at 6 days

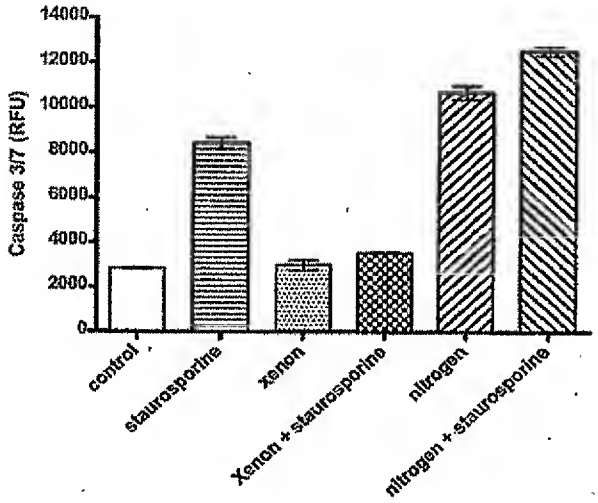
See, e.g., Hill et al., 2001, J. Biol. Chem. 276:25643-25646 (Exhibit A), page 25643, right column: "Staurosporine has been widely used as an inducer of apoptosis."

See also Bijur et al., 2000, J. Biol. Chem. 275:7583-7590 (Exhibit B), page 7586, right column: "Staurosporine is one of the most commonly used agents to experimentally induce apoptosis, and apoptosis occurs in essentially all cell types exposed to appropriate concentrations of staurosporine."

Xenon almost completely inhibits the activity of caspases 3 and 7

Figure 4 demonstrates that xenon almost completely inhibits the activity of caspases 3 and 7. Comparison of the fourth bar from the left (representing caspase 3 and 7 activity in the

presence of xenon and staurosporine) with the first bar from the left (representing background caspase 3 and 7 activity) indicates that xenon prevents the level of caspase 3 and 7 activity after treatment with staurosporine from rising more than an insignificant amount above the background level. This can be contrasted with the second bar from the left, which shows that caspase 3 and 7 activity rises greatly after staurosporine treatment in the absence of xenon.



Caspases 3 and 7 have a central role in apoptosis

Caspases, and in particular caspases 3 and 7, are among the key players in apoptosis and are responsible for many of the events that occur during programmed cell death. The measurement of their activity serves as a biochemical marker for apoptosis.

See, e.g., Taylor et al., Nature Reviews (Molecular Cell Biology), 2008, 231-241 (Taylor), page 233:

> Irrespective of the actual route to caspase activation, all pathways lead to the activation of the major effector caspases, caspase-3, caspase-6 and caspase-7, and

> these enzymes carry out much of the proteolysis that is seen during the demolition phase of apoptosis.
>
> See also Taylor, page 236:
>
> Caspases also target many proteins that are involved in essential housekeeping functions within the cell. Proteins that function in transcription (for example, nuclear factor of activated T cells (NFAT), nuclear factor-κB (NFκB) p50 and p65, and La ribonucleoprotein) and translation (for example, the translation initiation factors eIF2a, eIF3, eIF4 and the β-subunit of the nascent polypeptide-associated complex (βNAC)) come under caspase-mediated attack during apoptosis and ribosomal RNA is also degraded. Genomic DNA becomes extensively hydrolysed and the Golgi, ER and mitochondrial networks undergo fragmentation. Indeed, all of the major cell organelles become extensively remodelled during and, once again, caspases orchestrate much of this (Fig. 3). [citations omitted]

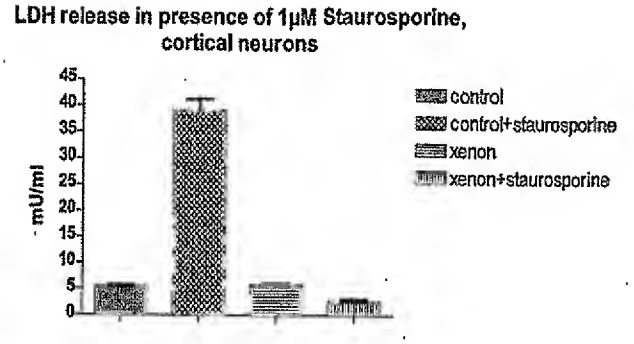
See also Bijur et al., 2000, J. Biol. Chem. 275:7583-7590 (Exhibit B), page 7584, left column: "The enzymes that ultimately carry out the command for apoptosis are the cysteine proteases known as caspases. ... [T]he measurement of caspase-3 activity can serve as a biochemical marker for the execution phase of apoptosis."

The effects of xenon were demonstrated in two very different cell types

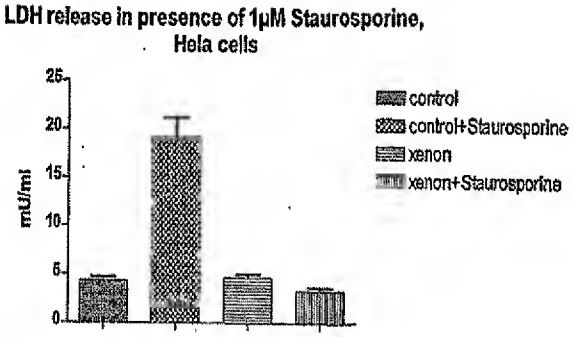
The present application contains experimental data from cortical neurons and HeLa cells. These are two very different types of cells: neurons are derived from brain and HeLa cells were obtained from cervical tissue. Yet xenon completely blocked apoptosis in both cell types. See Example 2 (neurons) and Example 3 (HeLa cells). That xenon has such dramatic effects in widely disparate cell types argues that xenon would be useful for the treatment of apoptosis-related conditions in a wide variety of cell types.

Xenon completely protected against apoptosis

The protective effect of xenon was essentially complete. See Figure 1 (cortical neurons)



and Figure 2 (HeLa cells)



Cell death that occurs during the circumstances recited in the claims is known to be largely due to apoptosis

The death of cells that occurs when tissues or organs are transplanted, after eye laser surgery, or during sepsis is largely brought about by apoptosis. See the specification, at pages 3-6.

The demonstration that xenon blocks two key components of apoptosis and that xenon acts on widely different cell types allows for a reasonable expectation that xenon will be useful to treat diseases or conditions where aberrant apoptosis is involved, including those recited in the present claims.

In view of the above, it is respectfully requested that this rejection be withdrawn.

The rejection under 35 U.S.C. §102(b)

Claims 12 and 16-24 were rejected for anticipation in view of International Patent Publication WO 00/53192 (the '192 application).

Claims 12 and 20-24 have been canceled. Claims 16-19 have been amended so as to depend from claims 13-15, which were not subjected to this rejection. Accordingly, it is respectfully requested that this rejection be withdrawn.

Claims 12, 16, and 18-23 were rejected for anticipation in view of U.S. Patent No. 6,274,633 (Franks).

Claims 12 and 20-23 have been canceled. Claims 16, 18, and 19 have been amended so as to depend from claims 13-15, which were not subjected to this rejection. Accordingly, it is respectfully requested that this rejection be withdrawn.

The time for responding to the Office Action was set for January 8, 2009. Therefore, it is believed that this response is timely. If this is in error, please treat this response as containing a Petition for the Extension of Time under 37 C.F.R. § 1.136(a) for a period sufficient to permit the filing of this paper and charge any corresponding fees to Kenyon & Kenyon's Deposit Account No. 11-0600.

The Applicants hereby make a Conditional Petition for any relief available to correct any defect seen in connection with the filing of this paper, or any defect seen to be remaining in this application after the filing of this paper. The Commissioner is authorized to charge Kenyon & Kenyon's Deposit Account No. 11-0600 for the Petition fee and any other fees required to effect this Conditional Petition.

Respectfully Submitted,

Date: January 6, 2009

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Insulin-stimulated Protein Kinase B Phosphorylation on Ser-473 Is Independent of Its Activity and Occurs through a Staurosporine-insensitive Kinase*

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Full activation of protein kinase B (PKB, also called Akt) requires phosphorylation on two regulatory sites, Thr-308 in the activation loop and Ser-473 in the hydrophobic C-terminal regulatory domain (numbering for PKBα/Akt-1). Although 3′-phosphoinositide-dependent protein kinase 1 (PDK1) has now been identified as the Thr-308 kinase, the mechanism of the Ser-473 phosphorylation remains controversial. As a step to further characterize the Ser-473 kinase, we examined the effects of a range of protein kinase inhibitors on the activation and phosphorylation of PKB. We found that staurosporine, a broad-specificity kinase inhibitor and inducer of cell apoptosis, attenuated PKB activation exclusively through the inhibition of Thr-308 phosphorylation, with Ser-473 phosphorylation unaffected. The increase in Thr-308 phosphorylation because of overexpression of PDK1 was also inhibited by staurosporine. We further show that staurosporine (CGP 39360) potently inhibited PDK1 activity *in vitro* with an IC_{50} of ~0.22 μM. These data indicate that agonist-induced phosphorylation of Ser-473 of PKB is independent of PDK1 or PKB activity and occurs through a distinct Ser-473 kinase that is not inhibited by staurosporine. Moreover, our results suggest that inhibition of PKB signaling is involved in the proapoptotic action of staurosporine.

Protein kinase B (PKB)[1] is activated by growth factors in a phosphoinositide 3-kinase (PI3K)-dependent manner, through translocation to the plasma membrane and phosphorylation on two regulatory sites, Thr-308 in the activation loop in the kinase domain and Ser-473 in the hydrophobic C-terminal regulatory domain (1). Phosphorylation on both sites are required for full activation of PKB; however, the contribution of each site toward PKB activation is not equal. Thus, whereas phosphorylation on Thr-308 alone is able to increase PKB activity, phosphorylation on Ser-473 alone does not significantly stimulate the kinase (1, 2). Although the upstream kinase responsible for phosphorylation of Thr-308 has been identified as 3′-phosphoinositide-dependent kinase-1 (PDK1), the identity of the Ser-473 kinase has yet to be determined (3, 4).

Mitogen-activated protein kinase-activated protein kinase-2 was the first kinase shown to phosphorylate PKBα on Ser-473 *in vitro* (1). However, mitogen-activated protein kinase-activated protein kinase-2 is unlikely to be the physiological Ser-473 kinase as it is activated downstream of p38 mitogen-activated protein kinase in response to stress, in a PI3K-independent manner, and inhibition of p38 mitogen-activated protein kinase by SB 203580 did not interfere with activation of PKB (1). Integrin-linked kinase (ILK) was also shown to phosphorylate Ser-473 on PKBα *in vitro*, and overexpression of a kinase-inactive ILK inhibited Ser-473 phosphorylation (5). However, certain kinase-inactive ILK mutants also induced Ser-473 phosphorylation, suggesting that ILK is unlikely to be the direct Ser-473 kinase *in vivo* (6). Two further candidate Ser-473 kinases have been proposed recently, PDK1 (7) and PKB itself (8). PDK1, in the presence of a peptide resembling the phosphorylated Ser-473 region of PKB, is able to phosphorylate Ser-473, in addition to Thr-308 of PKBα (7). However, PDK1 is clearly not the *in vivo* Ser-473 kinase, as PDK1-null embryonic stem cells are impaired in Thr-308 but not Ser-473 phosphorylation (9). Autophosphorylation was originally ruled out, because kinase-inactive PKBα undergoes insulin-like growth factor-1 (IGF-1)-induced phosphorylation at both Thr-308 and Ser-473 when overexpressed in human embryonic kidney (HEK) 293 cells (1). In contrast to these observations, Toker and Newton (8) recently demonstrated that IGF-1 stimulated phosphorylation of kinase-inactive PKBα on Thr-308 but not on Ser-473 when overexpressed in the same cells and that PKBα is able to autophosphorylate on Ser-473 *in vitro* (8). Thus, it seems possible that agonist-induced Ser-473 phosphorylation may be mediated by PKB itself.

To further characterize the upstream kinase(s) involved in the activation of PKB, we have adopted a pharmacological approach by screening for protein kinase inhibitors that differentially inhibit either Thr-308 or Ser-473 phosphorylation. We found that staurosporine, a broad-specificity protein kinase inhibitor, attenuated PKB activation specifically through inhibition of PDK1, with an IC_{50} of ~0.22 μM *in vitro*. Staurosporine has been widely used as an inducer of apoptosis; however, the cellular target(s) of its proapoptotic action are not known. Our data suggest that at least part of the apoptotic effects of staurosporine is due to inhibition of PKB signaling. In contrast to Thr-308 phosphorylation, insulin-stimulated phosphorylation of the Ser-473 site was not reduced by staurosporine treatment (up to 1 μM). Taken together, our results suggest that

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[1] The abbreviations used are: PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; PDK1, 3′-phosphoinositide-dependent protein kinase 1; ILK, integrin-linked kinase; IGF-1, insulin-like growth factor-1; HEK, human embryonic kidney; PKC, protein kinase C; GST, glutathione *S*-transferase; PH, pleckstrin homology; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HA, hemagglutinin; m/p, myristoylated/palmitylated.

phosphorylation of PKB on Ser-473 does not occur by autophosphorylation but rather through the action of an upstream kinase that is resistant to staurosporine and distinct from PDK1.

EXPERIMENTAL PROCEDURES

Expression Constructs and Transfection of Cells—Culture and transfection of HEK293 cells and the expression constructs used in this study have been described previously (1, 10–12).

Recombinant Proteins—Expression and infection of insect Sf9 cells have been described previously for PKB and PKC (13, 14). Human PDK1-glutathione *S*-transferase fusion protein (GST-PDK1) was cloned in a modified pFastBac vector (Life Technologies, Inc.) and prepared as described previously (13).

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assays—Cell lysis, immunoprecipitation, immunoblotting, and PKB assay using Crosstide peptide (GRPRTSSAEG) were performed as described previously (11). Phospho-specific PKB antibodies were purchased from Cell Signaling Technologies. In addition, we also produced and purified an anti-phospho-Ser-473 PKB antibody using the peptide Arg-Pro-His-Pbe-Pro-Gln-Phe-Ser(PO_3H_2)-Tyr-Ser-Ala-Ser (15). Assays for recombinant PKCα and PKCζ have been described previously (14). Recombinant GST-PDK1 was similarly assayed, using 0.1 mg/ml casein (Sigma) as substrate.

RESULTS

We have previously reported the characterization of a PKBα construct in which the pleckstrin homology (PH) domain was replaced by the C1 domain of PKC (C1-PKBα-ΔPH) (11). C1-PKBα-ΔPH translocated to the membrane upon stimulation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and was activated and phosphorylated on both Thr-308 and Ser-473 (11). TPA-stimulated activation of C1-PKBα-ΔPH was inhibited by PI3K inhibitors, as well as the broad range protein kinase inhibitor, staurosporine (11). Interestingly, whereas the former inhibited phosphorylation on both sites, staurosporine treatment specifically attenuated phosphorylation on Thr-308 without affecting Ser-473 phosphorylation (11).

To extend our observations, the effect of a panel of protein kinase inhibitors was compared with staurosporine (Fig. 1). Staurosporine obtained from Alexis or Novartis (CGP 39360) inhibited TPA-stimulated Thr-308 phosphorylation and activation of C1-PKBα-ΔPH, without affecting Ser-473 phosphorylation (Fig. 1*B*). CGP 39360 was more potent than staurosporine (Alexis), requiring 0.1 and 1 μM to reduce kinase activity and Thr-308 phosphorylation to basal levels, respectively (Fig. 1*B*). This difference may be because of improved purity of the chemical produced by Novartis. The staurosporine derivative CGP 41251 also inhibited Thr-308 phosphorylation and activation of C1-PKBα-ΔPH but was much less potent than CGP 39360 (Fig. 1*B*). The inactive analog of CGP 41251, CGP 42700, had no significant effect (Fig. 1*B*). Three other protein kinase inhibitor compounds examined (CGP 25956, CGP 45910, and CGP 57148B) had an inhibitory effect only at concentrations above 10 μM, where they reduced phosphorylation at both sites to below basal levels (Fig. 1*C*). Notably, an effect of CGP 57148B (STI571 or Glivec) was only observed at 40 μM (Fig. 1*C*). CGP 57148B is a potent inhibitor of Abl and PDGF receptor tyrosine kinases that selectively inhibits the growth of Bcr/Abl-transformed cells (16) and is now in clinical trials for treatment of chronic myeloid leukemia. The effects of CGP 57148B were observed concentrations <10 μM, and our results show that it does not significantly affect the PDK1/PKB pathway at this concentration (Fig. 1*C*).

To extend our observations to wild type PKB, we examined the effect of staurosporine on insulin-stimulated activation of HA-PKBα expressed in HEK293 cells. Staurosporine treatment inhibited insulin-stimulated HA-PKBα activation in a dose-dependent manner, with complete inhibition observed at 1 μM (Fig. 2*A*). Similar to C1-PKBα-ΔPH, this inhibitory effect of staurosporine on HA-PKBα activity correlated with an inhibition of Thr-308 phosphorylation (Fig. 2*A*). In contrast, phosphorylation on Ser-473 was slightly enhanced with increasing concentrations of staurosporine (Fig. 2*A*). A similar inhibitory effect of staurosporine was observed for insulin-stimulated Thr-308 phosphorylation of endogenous PKB in HEK293 cells (Fig. 2*B*). Two other modes of PKB activation were also examined, coexpression of PDK1 and constitutive membrane targeting. In agreement with previous results (3), coexpression of PDK1 with HA-PKBα resulted in a 3-fold increase in basal PKBα activity, together with constitutive phosphorylation of Thr-308 (Fig. 2*C*). Treatment with staurosporine reduced Thr-308 phosphorylation and kinase activity (Fig. 2*C*). Interestingly, overexpression of PDK1 also induced an increase in Ser-473 phosphorylation, reaching ~10% of the insulin-stimulated levels, that was reduced with staurosporine treatment, suggesting that it occurs through a mechanism different from insulin-stimulated Ser-473 phosphorylation (Fig. 2*C*). Targeting of PKB to the plasma membrane using the Lck myristoylation/palmitylation signal (m/p-PKBα) results in the constitu-

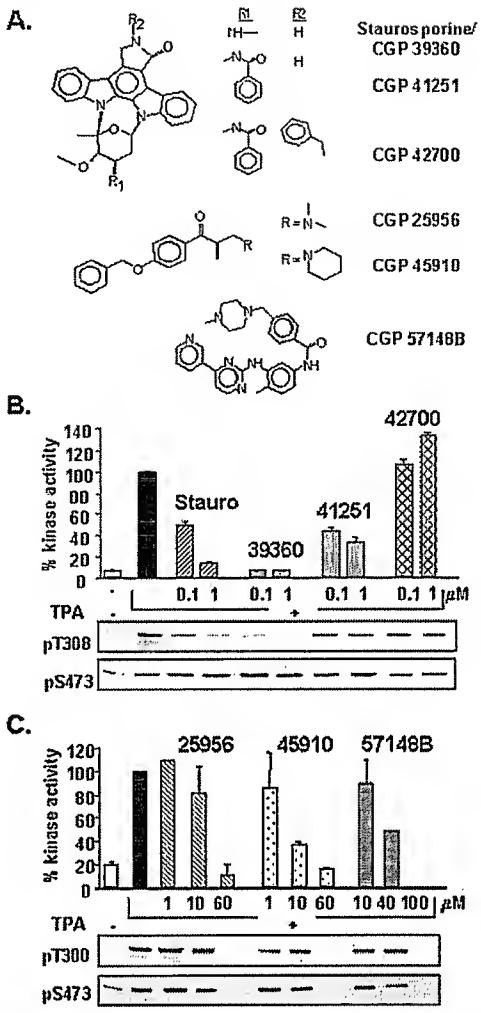

FIG. 1. Effect of staurosporine and its derivatives on TPA-stimulated activation of C1-PKBα-ΔPH. HEK293 cells transiently transfected with HA-C1-PKBα-ΔPH were treated with the indicated concentration of staurosporine (*Stauro*) (Alexis), CGP 39360, CGP 41251, CGP 42700 (*B*), CGP 25956, CGP 45910, or CGP 57148B/STI571 (*C*) for 30 min prior to stimulation with TPA (200 ng/ml; Life Technologies, Inc.) for 15 min. HA-C1-PKBα-ΔPH was immunoprecipitated and assayed for kinase activity or analyzed by immunoblotting with phospho-specific antibodies. Structures of the compounds used are shown in *A*.

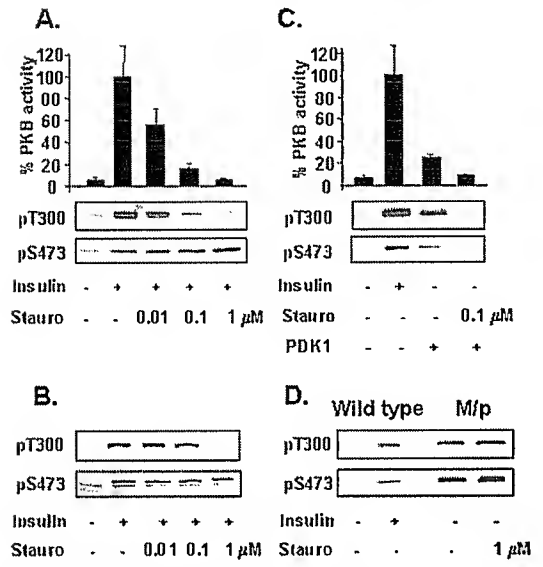

FIG. 2. **Differential effects of staurosporine on PKB phosphorylation induced by insulin, coexpression of PDK1, or membrane targeting.** HEK293 cells were transiently transfected with wild type HA-PKBα (*A*, *C*, and *D*), myristoylated/palmitylated HA-PKBα (*D*), and myc-PDK1 (*C*). Cells were treated with the indicated concentration of staurosporine (*Stauro*) for 30 min prior to stimulation with 0.1 μM insulin (Life Technologies, Inc.) for 15 min. HA-PKBα was immunoprecipitated and assayed for kinase activity or analyzed by immunoblotting with phospho-specific antibodies. The phosphorylation status of endogenous PKB was determined by immunoblotting cell lysates (20 μg) with phospho-specific antibodies (*B*).

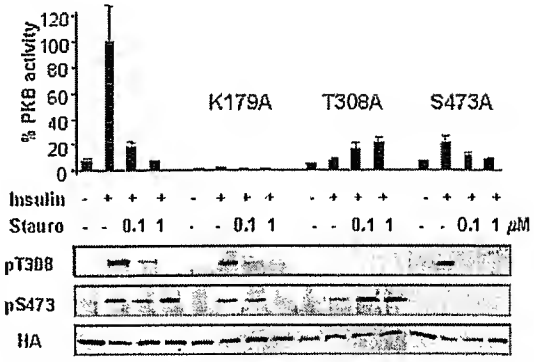

FIG. 3. **Effect of staurosporine on insulin-stimulated phosphorylation and activation of PKBα mutants.** HEK293 cells transiently transfected with wild type or mutant HA-PKBα were treated with 0.1 or 1 μM staurosporine (*Stauro*) for 30 min prior to stimulation with 0.1 μM insulin for 15 min. HA-PKBα expression was determined by blotting with an anti-HA antibody. PKB activity and phosphorylation was analyzed as for Fig. 1.

tive activation and phosphorylation of PKB (10). In contrast to Thr-308 phosphorylation induced by insulin or coexpression of PDK1, staurosporine did not reduce Thr-308 phosphorylation of m/p-PKBα (Fig. 2*D*). This observation indicates that dephosphorylation of PKB does not occur readily at the plasma membrane and that the phosphorylation step is the target of staurosporine.

Staurosporine is a competitive inhibitor that is thought to bind in the ATP pocket of target protein kinases (17). The observed effect on Thr-308 may occur via direct inhibition of PDK1, or staurosporine could bind to PKB, thus blocking the access to the phosphorylation site in the catalytic domain. To distinguish between these possibilities, we determined the inhibitory profiles of the CGP inhibitor compounds using recombinant GST-PDK1, GST-PKBα, PKCα, and PKCζ. CGP 39360 (staurosporine) was most potent against PKCα (IC_{50} < 3 nM) but also inhibited PDK1 (IC_{50} = 0.22 ± 0.09 μM), PKBα (IC_{50} = 0.83 ± 0.19 μM), and PKCζ (IC_{50} = 1.03 ± 0.37 μM) at higher concentrations. CGP 41251 selectively inhibited PKCα (IC_{50} = 0.04 ± 0.02 μM) and also inhibited PDK1 (IC_{50} = 1.72 ± 0.21 μM) but did not have significant effects on PKBα or PKCζ (up to 10 μM). These data suggest that the target of staurosporine and its derivative CGP 41251 is PDK1 rather than PKB. CGP 42700, CGP 25956, CGP 45910, and CGP 57148B did not inhibit any of the four kinases tested.

To further examine the regulation of PKB activation by upstream kinases, the effect of staurosporine on insulin-stimulated phosphorylation and activity of kinase-inactive (K179A) or phosphorylation-site mutants (T308A and S473A) of PKBα was examined (Fig. 3). In agreement with previously reported results (1), Thr-308 and Ser-473 phosphorylation occurred independently of each other upon insulin stimulation, as observed in the phosphorylation-site mutants (Fig. 3). In addition, the kinase-inactive mutant (K179A) was phosphorylated on both Thr-308 and Ser-473 upon insulin stimulation (Fig. 3). Staurosporine treatment inhibited insulin-stimulated Thr-308 phosphorylation on wild type, K179A, and S473A PKBα (Fig. 3), but its effect on Ser-473 phosphorylation of the different PKBα constructs was somewhat varied. Staurosporine at 0.1 and 1 μM did not inhibit insulin-stimulated Ser-473 phosphorylation of wild type and T308A-PKBα but even enhanced their phosphorylation, which was more readily observed in the T308A mutant (Fig. 3). Interestingly, insulin-stimulated Ser-473 phosphorylation of kinase-inactive PKBα was inhibited by staurosporine at 1 μM but not at 0.1 μM (Fig. 3). As staurosporine is a broad specificity kinase inhibitor, it is possible that complex effects are observed because of inhibition of numerous kinases/pathways at higher doses.

DISCUSSION

Phosphorylation at Thr-308 and Ser-473 is required for full activation of PKBα. Although the Thr-308 kinase has been identified (PDK1), the Ser-473 kinase remains elusive. Most recently, it has been suggested that autophosphorylation may be the mechanism by which PKB is phosphorylated on Ser-473 and that the previously reported phosphorylation of kinase-deficient PKB at this site is due to the activity of endogenous PKB (8). Our previous results with m/p-PKBα (10) and C1-PKBα-ΔPH (11) suggest that both upstream kinases are present in a constitutively active form at the plasma membrane. As PKB is present largely in the cytosol prior to stimulation, it seems unlikely that PKB itself is the physiological Ser-473 kinase. More directly, here we show that pretreatment with 1 μM staurosporine abolished insulin-stimulated PKB activation of both transiently expressed and endogenous PKB, without affecting Ser-473 phosphorylation (Fig. 2). In addition, using phospho-specific antibodies, we have confirmed our previous results (1) that kinase-inactive PKBα can be phosphorylated on both Thr-308 and Ser-473 in response to insulin (Fig. 3). Taken together, these data do not support the hypothesis that phosphorylation on Ser-473 occurs via autophosphorylation or trans-phosphorylation. Rather, it confirms the existence of a distinct Ser-473 kinase that is constitutively active at the plasma membrane of quiescent cells.

One kinase that fulfills the above criteria is PDK1. Indeed, PDK1 has the ability to phosphorylate PKBα on Ser-473 in the presence of an exogenous peptide that resembles phosphorylated Ser-473 (7). However, PDK1 is not the physiological Ser-473 kinase, because PDK1-null embryonic stem cells are not impaired in Ser-473 phosphorylation in response to IGF-1 (9), and staurosporine inhibits PDK1 activity without affecting

insulin-stimulated Ser-473 phosphorylation (Fig. 2). Interestingly, overexpression of PDK1 in HEK293 cells not only induced constitutive phosphorylation of PKB at Thr-308 but also caused a slight elevation of Ser-473 phosphorylation (Fig. 2*C*). In this case, however, Ser-473 phosphorylation is dependent on PDK1 activity, as it is attenuated by staurosporine treatment (Fig. 2*C*), and overexpression of a kinase-inactive PDK1 mutant did not increase basal Ser-473 phosphorylation in HEK293 cells (data not shown). Thus, although PDK1 is not the physiological Ser-473 kinase, it likely plays a role in Ser-473 phosphorylation, with the nature of this interaction yet to be defined.

ILK has recently come to attention as a prime candidate kinase for Ser-473 phosphorylation (5). According to our observations, the Ser-473 kinase should be staurosporine-resistant. Unfortunately, we were unable to determine the effect of staurosporine on ILK as we have not been able to detect any significant kinase activity of overexpressed or endogenous ILK by autophosphorylation or on myelin basic protein (data not shown). Intriguingly, ILK possesses a hydrophobic motif similar to the Ser-473 site, and when this serine was mutated to an acidic residue to mimic phosphorylation, the ability of a kinase-inactive ILK to induce Ser-473 phosphorylation was rescued (6). It was recently shown that the hydrophobic phosphorylation site in p90 ribosomal S6 kinase acts as a docking site for the recruitment of PDK1 (18). Thus, it is possible that ILK mediates the colocalization of PKB with PDK1 and the Ser-473 kinase.

Staurosporine exhibits anti-proliferative properties on a wide range of mammalian cells, and its derivatives UCN-01, CGP 41251, Ro 31–8220, and PKC412 (19–22) are being examined as potential therapeutic agents for the treatment of cancer. Despite the common use of staurosporine as an inducer of apoptosis, the direct cellular target of staurosporine is not known. Our finding that staurosporine inhibits PDK1 activity raises the possibility that staurosporine and its derivatives may induce apoptosis by interfering with survival signaling mediated by PDK1. Indeed, it was recently shown that reduction of PDK1 expression by antisense oligonucleotides induced apoptosis (23). Apart from PKB, PDK1 also phosphorylates and activates other kinases that are involved in cell survival, including p70 ribosomal S6 kinase (12), p90 ribosomal S6 kinase (24), and serum- and glucocorticoid-inducible protein kinase (25). Thus the mechanisms of staurosporine-induced apoptosis needs to be readdressed in light of its effects on PDK1 activity.

In summary, we have demonstrated that staurosporine attenuates PKB activation through direct inhibition of PDK1 activity, without affecting insulin-stimulation of Ser-473 phosphorylation. These results strongly suggest that insulin-stimulated phosphorylation on Ser-473 is not dependent on the activity of PDK1 or PKB. Our data is consistent with a model in which phosphorylation on Thr-308 and Ser-473 occurs via two distinct upstream kinases that are constitutively active at the plasma membrane, and of these, only the Ser-473 kinase is staurosporine-resistant.

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Glycogen Synthase Kinase-3β Facilitates Staurosporine- and Heat Shock-induced Apoptosis

PROTECTION BY LITHIUM*

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The potential role of glycogen synthase kinase-3β in modulating apoptosis was examined in human SH-SY5Y neuroblastoma cells. Staurosporine treatment caused time- and concentration-dependent increases in the activities of caspase-3 and caspase-9 but not caspase-1, increased proteolysis of poly(ADP-ribose) polymerase, and induced morphological changes consistent with apoptosis. Overexpression of glycogen synthase kinase-3β to levels 3.5 times that in control cells did not alter basal indices of apoptosis but potentiated staurosporine-induced activation of caspase-3, caspase-9, proteolysis of poly(ADP-ribose) polymerase, and morphological changes indicative of apoptosis. Inhibition of glycogen synthase kinase-3β by lithium attenuated the enhanced staurosporine-inducecd activation of caspase-3 in cells overexpressing glycogen synthase kinase-3β. In cells subjccted to heat shock, caspase-3 activity was more than three times greater in glycogen synthase kinase-3β-transfected than control cells, and this potentiated response was inhibited by lithium treatment. Thus, glycogen synthase kinase-3β facilitated apoptosis induced by two experimental paradigms. These findings indicate that glycogen synthase kinase-3β may contribute to pro-apoptotic-signaling activity, that inhibition of glycogen synthase kinase-3β can contribute to anti-apoptotic-signaling mechanisms, and that the neuroprotective actions of lithium may be due in part to its inhibitory modulation of glycogen synthase kinase-3β.

Glycogen synthase kinase-3 (GSK-3)[1] was initially identified as a kinase that phosphorylates glycogen synthase (1). Subsequent studies have demonstrated that GSK-3 surpasses this function and plays a broad role in cellular metabolism, including contributions to signaling activities, growth, and differentiation (2). GSK-3β has been shown to phosphorylate numerous substrates, including several transcription factors such as c-jun, c-myc (3–5), and heat shock factor-1 (6), cytoskeletal proteins such as the microtubule-associated protein tau (7, 8), and the multifunctional protein β-catenin (9). Thus it is now evident that the activity of GSK-3β influences a wide variety of cellular functions, including multiple signaling systems.

Much still remains to be learned about the regulation of GSK-3β activity and its role as a modulator of signaling cascades that determine cell fate. Although often considered to be a constitutively active enzyme, GSK-3β can be both activated and inhibited. Activation has been shown to occur subsequent to phosphorylation of Tyr^{216} (10) and recently by transient increases in intracellular calcium (11). Inhibition of GSK-3β can be induced by activation of the Wnt pathway (12) or by agents that activate a signaling cascade that commences when growth factors or insulin bind to their respective receptors (see Ref. 13 for review), resulting in the recruitment and activation of phosphatidylinositol 3-kinase. Activated phosphatidylinositol 3-kinase catalyzes the production of phosphatidylinositol 3,4,5-trisphosphate, which binds the pleckstrin homology domain of Akt (also known as protein kinase B) to bring it into close proximity with phosphoinositide-dependent kinase-1. The juxtaposition of phosphoinositide-dependent kinase-1 to Akt on the membrane facilitates the phosphorylation and activation of Akt by phosphoinositide-dependent kinase-1 (14). Subsequently, Akt dissociates from the membrane and can phosphorylate Ser^{9} of GSK-3β, which inhibits its activity (15). Activation of the phosphatidylinositol 3-kinase/Akt-signaling pathway protects cells from pro-apoptotic stimuli as well as reducing the activity of GSK-3β. For example, activators of phosphatidylinositol 3-kinase and Akt, such as insulin-like growth factor-1, platelet-derived growth factor (16, 17), and interleukin-2 (18) and -3 (19, 20), protect cells from a variety of apoptotic insults. Thus, the signaling mechanism that is associated with inhibition of GSK-3β is also associated with anti-apoptotic outcomes. Akt-mediated cell protection has been attributed to processes other than inhibition of GSK-3β, such as by phosphorylation of the proapoptotic Bcl family member Bad (21) or by preventing the release of cytochrome c from mitochondria (22), but it is not known whether or not inhibition of GSK-3β contributes to the anti-apoptotic effects of Akt activity. There is some evidence of the converse, that activation of GSK-3β contributes to pro-apoptotic signaling, as it was recently found that overexpression of GSK-3β in Rat-1 and PC12 cells stimulated apoptosis (23). Considering the potentially important role of GSK-3β in regulating apoptosis, it was of great interest to note that lithium was recently discovered to inhibit of GSK-3β (24, 25). Lithium is used therapeutically for the treatment of bipolar disorder, and although it has been used in the psychiatric domain for many years, its influences at the biochemical level are only beginning to be elucidated (26). One of the most intriguing findings is that lithium confers protection to neurons against pro-apoptotic stimuli such as glutamate-induced excitotoxicity (27), C2-ceramide (28), radiation (29), and ischemia (30). Taken together, these findings raise

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[1] The abbreviations used are: GSK-3, glycogen synthase kinase-3; PDK-1, phosphoinositide-dependent kinase-1; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; Ac-, acetyl; AMC, amidomethylcoumarin; ANOVA, analysis of variance.

the possibilities that GSK-3β contributes to apoptotic-signaling cascades and that inhibition of GSK-3β contributes to the neuroprotective properties of lithium.

The enzymes that ultimately carry out the command for apoptosis are the cysteine proteases known as caspases. Caspases, which are zymogens, are typically cleaved autocatalytically or by other caspases from inactive procaspase proteins to produce activated enzymes (31, 32). Caspase-3, also called CPP32, is activated by many pro-apoptotic stimuli and is an early step in the execution phase of apoptosis (33). The activation of caspase-3 commences after apoptotic signals induce the release of cytochrome c from the mitochondrial intermembrane space (34), which subsequently associates with apoptotic protease-activating factor-1 and procaspase-9 to form the "apoptosome" (35). This complex formation stimulates the oligomerization of procaspase-9 and its autocatalytic activation. The effect of caspase-9 activity is the proteolytic activation of downstream caspases such as caspase-3 (36), which in turn proteolyzes the DNA-binding protein poly(ADP-ribose) polymerase (PARP) (37) and other proteins. Hence the measurement of caspase-3 activity can serve as a biochemical marker for the execution phase of apoptosis.

The goal of this investigation was to test if GSK-3β activity modulates apoptosis using the neuronal model system of human neuroblastoma SH-SY5Y cells. Apoptosis was generated using staurosporine, which previously has been demonstrated to induce apoptosis in these and other cells (38–40), and heat shock, a method widely used to cause cell stress (41, 42). GSK-3β activity was increased by overexpression, and GSK-3β activity was decreased by using lithium. The results show that overexpression of GSK-3β alone did not induce apoptosis, but it sensitized cells to apoptosis caused by exposure to staurosporine or to heat shock, and that inhibition of GSK-3β by lithium attenuated activation of caspase-3.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection of Cells—Human neuroblastoma SH-SY5Y cells were grown in continuous culture RPMI media (Cellgro, Herndon, VA) containing 10% horse serum (Life Technologies), 5% fetal clone II (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stably transfected cells were maintained with transfection-maintenance medium that contained 100 μg/ml G418 (Geneticin); other components were the same as continuous culture media. Cells were maintained in humidified, 37 °C incubators with 5% CO_2. Cells were plated at a density of 10^5 cells/60-mm dish 48 h before apoptosis-inducing treatments.

For stable transfection, SH-SY5Y cells were replated one day before electroporation. The cells were removed from the dish with continuous culture medium containing 0.05% of trypsin, diluted with continuous culture medium, and centrifuged for 5 min at 250 × *g*. Cells were washed, resuspended in 1 ml HEPES buffer (0.14 M NaCl, 25 mM HEPES, 0.075 mM Na_2HPO_4, pH was adjusted to 7.05 with 10 M NaOH), and incubated with 10 μg of HA-GSK-3β in pcDNA3.1 (generously provided by Dr. J. R. Woodgett, University of Toronto) on ice for 10 min. Electroporation was carried out with a Bio-Rad Pulse II electroporator set at 0.25 kV and 960 microfarads as described previously (43). After electroporation, cells were incubated on ice for 10 min and then mixed with 10 ml of continuous culture medium and plated onto a 100-mm Corning dish. After 48 h the medium was replaced with transfection-maintenance medium, and the cells were maintained in transfection-maintenance medium for approximately 1 month until only cells resistant to Geneticin survived. Cells were cloned and screened for expression of HA-GSK-3β.

Collection of Lysates—For immunoblotting, cells in 60-mm plates were washed twice with phosphate-buffered saline and were lysed with 100 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, and 0.2% Nonidet P-40). For caspase activity, cells were lysed with 100 μl of lysis buffer without sodium orthovanadate. The lysates were collected in microcentrifuge tubes, sonicated, and centrifuged. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) method (Pierce). The lysates were stored at −80 °C until used either for immunoblotting or measuring caspase activity.

Immunoblotting—Cell lysates were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were separated in 8% SDS-polyacrylamide gels for immunoblotting GSK-3β and caspase-9 or in 7.5% and 10% SDS-polyacrylamide gels for immunoblotting PARP and caspase-3, respectively. The proteins were transferred to nitrocellulose. Blots were probed with antibodies to GSK-3β, caspase-3, caspase-9, or PARP (PharMingen/Transduction Laboratories San Diego, CA) followed by incubation with horseradish peroxidase-conjugated secondary antibody. Blots were developed using peroxidase substrate chemiluminescence (Kirkegaard & Perry, Gaithersburg, MD) and analyzed by PhosphoImager (Molecular Dynamics).

GSK-3β Activity—The activity of GSK-3β was measured essentially as described previously (44, 45). Cells were lysed in immunoprecipitation lysis buffer (20 mM Tris, pH 7.5, 0.2% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 1 nM okadaic acid, 100 mM sodium fluoride, and 1 mg/ml glycogen). The lysates were sonicated in microcentrifuge tubes for 10 s on ice and centrifuged at 20,800 × *g* for 15 min. After the protein concentration was determined, 100 μg of protein (1 μg/μl) was precleared with 40 μl of protein G-Sepharose beads for 3 h at 4 °C, then incubated with 2 μg of monoclonal GSK-3β antibody (Pharmingen/Transduction Laboratories) overnight at 4 °C with gentle agitation. The immobilized immune complexes were washed three times with immunoprecipitation lysis buffer and once with 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol. Kinase activity was assayed in a total volume of 15 μl of kinase buffer containing 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 250 μM ATP, 1.4 μCi of [γ-^{32}P]ATP, and 100 μM phosphoglycogen synthase peptide-2 (YRRAAVPPSPSLSRHSSPHQSEDEEE) (Upstate Biotechnology, Inc., Lake Placid, NY). Glycogen synthase (Ala21) peptide-2 was used as negative control. The samples were incubated at 30 °C for 30 min, the reaction tubes were centrifuged for 1 min, and triplicate 5-μl aliquots of reaction supernatants were spotted onto 1 cm × 2 cm P81 filter paper. The filter papers were washed 4 times in 0.5% phosphoric acid for a total time of 1 h, rinsed in 95% ethanol, air-dried, and counted in a liquid scintillation counter. The efficiency of GSK-3β immunoprecipitation was determined by immunoblotting for GSK-3β.

Caspase Activity—Fluorometric assays were conducted in 96-well clear-bottom plates, and all measurements were carried out in triplicate wells. To each well 200 μl of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol) was added. Peptide substrates for caspase-3 (Ac-DEVD-AMC), caspase-9 (Ac-LEHD-AMC), or caspase-1 (Ac-YVAD-AMC) (Alexis Biochemicals, San Diego, CA) were added to each well to a final concentration of 25 ng/μl. When the caspase-3 inhibitor (Ac-DEVD-CHO) was used, it was added at a concentration of 2.5 ng/μl immediately before the addition of the caspase-3 substrate. Cell lysates (20 μg of protein) were added to start the reaction. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer without the cell lysate. Assay plates were incubated at 37 °C for 1 h for measurement of caspase-3 and 3 h for measurement of caspase-1 and caspase-9, based on preliminary measurements of the time course (0.5 to 6 h) of caspase activities. Fluorescence was measured on a fluorescence plate reader (Bio-Tek, Winooski, VT) set at 360 nm excitation and 460 nm emission. Caspase activity was calculated as ((mean AMC fluorescence from triplicate wells) − (background fluorescence))/μg of protein.

Nuclear Staining—Cells were cultured on poly-D-lysine-coated glass coverslips placed in 35-mm culture plates. After treatments, medium was removed, and the cells were fixed in 2% paraformaldehyde for 1 h at room temperature. After two washes with phosphate-buffered saline, cells were stained with 10 ng/μl Hoechst 33342 (Molecular Probes, Eugene, OR) for 1 h at room temperature. The coverslips were rinsed twice with phosphate-buffered saline and then mounted onto glass slides using Immu-Mount (Shandon, Pittsburgh, PA). The slides were examined by fluorescence microscopy (Nikon) set at 400× magnification. To score the number of cells exhibiting apoptotic morphology, a minimum of 300 cells were counted/coverslip.

RESULTS

Induction of Caspase-3 Activity and Apoptosis—SH-SY5Y cells were treated with staurosporine and then examined for characteristics associated with apoptosis, including activation of caspases, proteolysis of PARP, and changes in cellular mor-

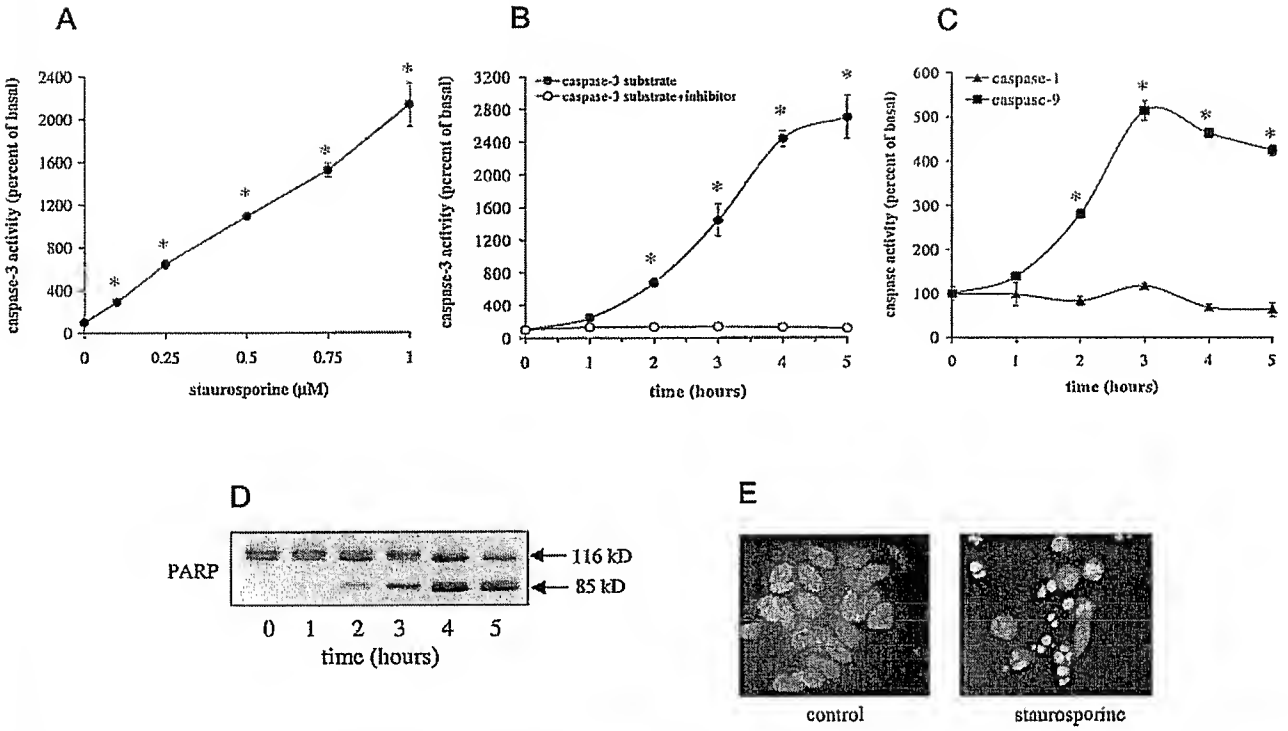

FIG. 1. **Staurosporine-induced apoptosis.** SH-SY5Y cells were treated with 0.1, 0.25, 0.5, 0.75, or 1 μM staurosporine for 3 h (*A*) or with 0.5 μM staurosporine for 1–5 h (*B*). Caspase-3 activity in cell lysates was measured with DEVD-AMC, a caspase-3 substrate, (*A*) or with DEVD-AMC with or without DEVD-CHO, a caspase-3 inhibitor, as described under "Experimental Procedures" (*B*). *C*, caspase-9 and caspase-1 activities in cell lysates were measured with LEHD-AMC, a caspase-9 substrate, or with YVAD-AMC, a caspase-1 substrate. Values are expressed as a percent of caspase activity in untreated cells. Means ± S.E., $n = 3$ experiments; $^*p < 0.05$ (ANOVA) compared with untreated cells. *D*, PARP proteolysis was measured in cell lysates by immunohlot analysis. *E*, apoptotic morphology was examined with Hoechst 33342 stain of untreated cells and cells treated with 0.5 μM staurosporine for 8 h. A representative section is shown at 400× magnification.

phology. Caspase-3 activity increased concentration dependently after treatment with 0.1 to 1 μM staurosporine (Fig. 1*A*). Examination of the time-dependent activation of caspases induced by 0.5 μM staurosporine revealed that caspase-3 activity began to increase approximately 1 h after staurosporine treatment and reached maximum activation after 4–5 h (Fig. 1*B*). The specificity of the caspase-3 assay was confirmed by the use of a caspase-3 inhibitor, which resulted in the complete inhibition of caspase-3 activity. Caspase-9 and caspase-1 were also evaluated to test if other caspases were activated by staurosporine treatment. Caspase-9 activity began to increase after 1 h of staurosporine treatment and reached a maximum within 3 h (Fig. 1*C*), but caspase-1 was not activated. The time-dependent proteolytic cleavage of PARP to the 85-kDa fragment after treatment with 0.5 μM staurosporine corresponded to the time-dependent activation of caspase-3 (Fig. 1*D*). Examination of cells treated with 0.5 μM staurosporine for 8 h and stained with Hoechst 33342 revealed the characteristic morphology associated with apoptosis, such as nuclear condensation and cell shrinkage (Fig. 1*E*).

Effect of Modulating GSK-3β on Staurosporine-induced Caspase-3 Activation—To test the hypothesis that GSK-3β facilitates apoptosis, SH-SY5Y cells were transfected with HA-tagged GSK-3β, and several stable cell lines were established. Fig. 2 shows the levels of GSK-3β, procaspase-3, and procaspase-9 in untransfected control cells, vector-transfected cells, and four cell lines of HA-GSK-3β-overexpressing cells. Two bands are evident on the GSK-3β immunoblots because HA-GSK-3β migrates slower than endogenous GSK-3β due to the HA-tag (Fig. 2*A*). In these four cell lines total GSK-3β averaged 347% ± 7% and 379% ± 7% of the GSK-3β in untransfected cells and vector-transfected cells, respectively (Fig. 2*B*). The activity of GSK-3β was also measured in each cell line (Fig. 2C). In the four GSK-3β-transfected cell lines, GSK-3β activity averaged 327% ± 57% and 349% ± 61% of the GSK-3β in untransfected and vector-transfected cells, respectively. In GSK-3β-overexpressing cells, the levels of procaspase-3 averaged 90% ± 11% and 113% ± 13% (Fig. 2*B*), and the level of procaspase-9 averaged 111% ± 10% and 95% ± 9% that of the levels of these caspases in untransfected cells and vector-transfected cells, respectively. Further experiments were carried out using cell line 7, and results were confirmed using the other three HA-GSK-3β-expressing cell lines.

The staurosporine concentration-dependent activation of caspase-3 was measured 3 h after treatment in vector-transfected cells and in the four subclones of HA-GSK-3β-transfected cells. Basal caspase-3 activities in vector- and HA-GSK-3β-transfected cells were not different from control SH-SY5Y cells. In vector-transfected cells, activation of caspase-3 by staurosporine (Fig. 3*A*) was equivalent to that obtained in untransfected SH-SY5Y cells (Fig. 1). In marked contrast, overexpression of HA-GSK-3β greatly potentiated staurosporine-induced activation of caspase-3 in all four cells lines overexpressing GSK-3β. The caspase-3 activity in HA-GSK-3β-transfected cells averaged 280% and 250% of that in vector-transfected cells after treatment with 0.1 and 0.5 μM staurosporine, respectively (Fig. 3*A*). Furthermore, caspase-9 activity in HA-GSK-3β-transfected cells averaged 180% and 245% of that in vector-transfected cells after treatment with 0.1 and 0.5 μM staurosporine, respectively (Fig. 3*B*). The potentiation of staurosporine-induced caspase-3 activity by GSK-3β overexpression was confirmed by a greater degree of PARP

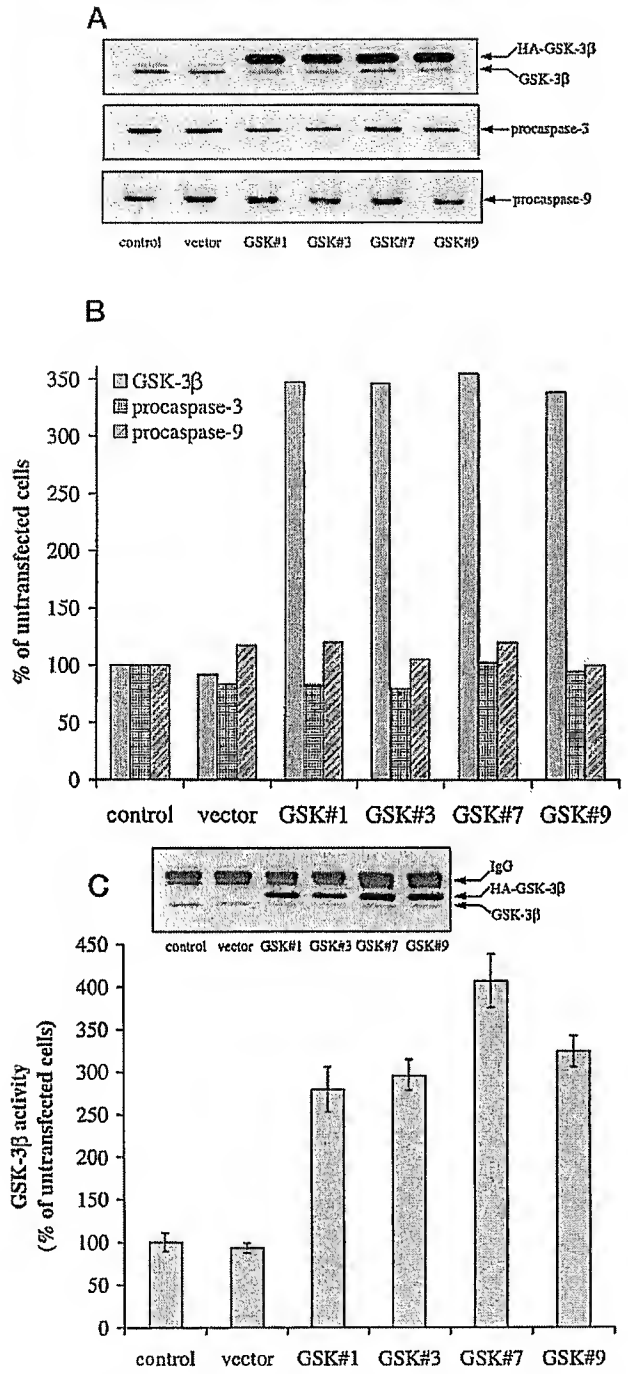

FIG. 2. **GSK-3β overexpression in SH-SY5Y cells.** Proteins from control SH-SY5Y cells, from vector-transfected SH-SY5Y cells, or four different clones of SH-SY5Y cells stably transfected with HA-GSK-3β were immunoblotted for GSK-3β, procaspase-3, or procaspase-9 (*A*), and protein bands were quantitated and are expressed as the percent of levels in control SH-SY5Y cells (*B*). Values are the average of a minimum of two experiments. *C*, GSK-3β activity was measured as described under "Experimental Procedures." Values are the means ± S.D. of the GSK-3β activity in control, untransfected cells (100% represents 11,747 cpm) of triplicate measurements. The *inset* shows immunoblots of GSK-3β immunoprecipitated from each cell line.

proteolysis (Fig. 3*C*) and greater numbers of cells exhibiting apoptotic morphology (Fig. 3, *D* and *E*). These results indicate that overexpression of HA-GSK-3β increases the sensitivity of cells to staurosporine-induced apoptosis.

Lithium Attenuates Staurosporine-induced Caspase-3 Activation—Cells were pretreated with lithium to inhibit GSK-3β to test if inhibition of GSK-3β attenuates staurosporine-induced activation of caspase-3 in control or HA-GSK-3β-transfected cells. The large differences in the concentration of staurosporine that activated caspase-3 in control and HA-GSK-3β-transfected cells made it necessary to use different protocols for each cell line, so caspase-3 was measured in untransfected cells 4 h after treatment with 0.5 μM staurosporine and in HA-GSK-3β-transfected cells 3 h after treatment with 0.1 μM staurosporine. Untransfected SH-SY5Y cells were pretreated with 1.25, 2.5, and 5 mM lithium for 24 h and then treated with 0.5 μM staurosporine for 4 h. Pretreatment of control SH-SY5Y cells with 5 mM lithium significantly decreased staurosporine-induced caspase-3 activity by 28% (Fig. 4*A*). HA-GSK-3β-transfected cells were pretreated for 24 h (Fig. 4*B*) or were treated chronically for 7 days (Fig. 4*C*) with 1.25, 2.5, or 5 mM lithium. Pretreatment for 24 h with 5 mM lithium significantly reduced the staurosporine-induced caspase-3 activity by 40%. Chronic treatment with 1.25, 2.5, and 5 mM lithium resulted in reductions of 23%, 31%, and 65%, respectively, of caspase-3 activity induced by staurosporine. These results demonstrate that the facilitatory effect of GSK-3β on staurosporine-induced caspase-3 activity is attenuated by lithium.

GSK-3β Potentiates Heat Shock-induced Activation of Caspase 3—To test if the potentiation by HA-GSK-3β and attenuation by lithium of caspase-3 activation occur with another apoptotic paradigm, the effects of these treatments were measured in cells subjected to heat shock (30 min at 45 °C) followed by incubation at 37 °C, a model widely used to study the responses of cells to stress (41, 42). In vector-transfected cells caspase-3 activity increased to 425% and 975% that of the basal activity following 30 min and 90 min of incubation at 37 °C, respectively (Fig. 5*A*). In HA-GSK-3β-transfected cells, caspase-3 activity increased to 1500% and 3100% that of the basal activity after 30 and 90 min of incubation at 37 °C, respectively. Pretreatment of HA-GSK-3β-transfected cells with 1.25, 2.5, and 5 mM Li for 24 h reduced the heat shock-induced caspase-3 activation by 13%, 25%, and 56%, respectively. These results demonstrate that overexpression of GSK-3β potentiates the activation of caspase-3 after treatment with heat shock and that pretreatment with lithium, a GSK-3β inhibitor, attenuates this effect.

DISCUSSION

In marked contrast to its modest beginning as a regulator of glycogen synthesis, GSK-3β has been found to participate in a remarkable number of signaling pathways, which, based on the findings reported here, include the ultimate decision between cell death and survival. Thus, cells overexpressing GSK-3β succumbed much more rapidly to the apoptosis-inducing actions of staurosporine or exposure to elevated temperature than did cells with a normal complement of GSK-3β. Furthermore, lithium, an inhibitor of GSK-3β, counteracted the facilitation of apoptosis caused by overexpression of GSK-3β. These findings demonstrate that initial steps in the apoptotic signaling involving activation of caspases can be influenced by GSK-3β.

Staurosporine is one of the most commonly used agents to experimentally induce apoptosis, and apoptosis occurs in essentially all cell types exposed to appropriate concentrations of staurosporine, suggesting that it activates a cell death program common to all cells (38). Staurosporine previously has been reported to induce apoptosis in human neuroblastoma SH-SY5Y cells (40, 46). In these cells, 0.5 μM staurosporine was reported to increase caspase-3 activity, result in PARP proteolysis, and cause morphological changes indicative of apoptosis

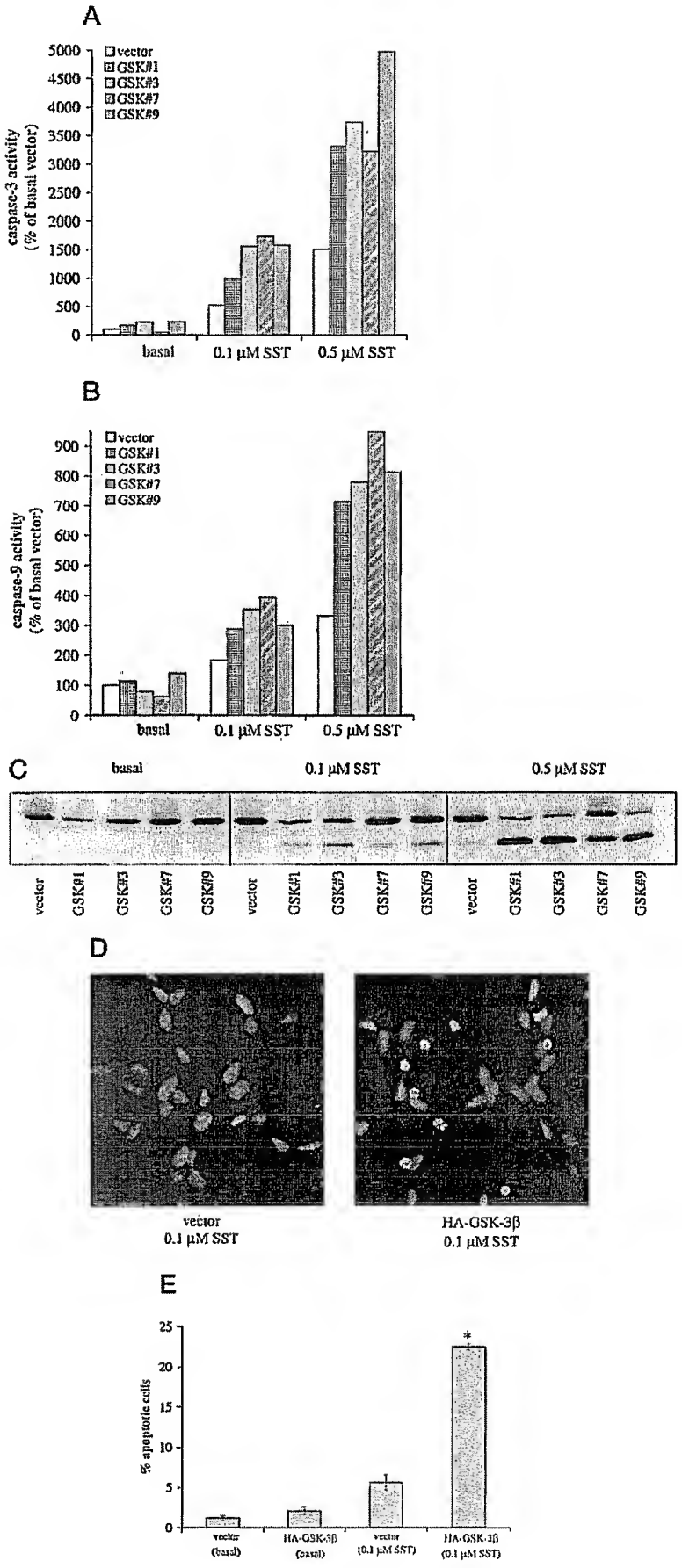

FIG. 3. **Effects of GSK-3β overexpression on staurosporine-induced apoptosis.** Cells transfected with HA-GSK-3β or vector were treated with 0 (basal), 0.1, or 0.5 μM staurosporine (*SST*) for 3 h followed by measurement of caspase-3 activity (*A*) or caspase-9 activity as described under "Experimental Procedures" (*B*). Values are expressed as a percent of activity in untreated vector-transfected cells. *C*, PARP proteolysis was analyzed by SDS-PAGE and immunoblotting for PARP. *D*, apoptosis morphology was examined in vector- and HA-GSK-3β-transfected cells treated with 0.1 μM staurosporine for 8 h and stained with Hoechst 33342 as described under "Experimental Procedures." A representative section is shown at 400× magnification. *E*, to quantitate the number of cells exhibiting apoptosis, a minimum of 300 cells were counted per coverslip. Mean ± S.E., $n = 3$ experiments; $^*p < 0.05$, comparing vector-transfected and HA-GSK-3β-transfected cells treated with 0.1 μM staurosporine for 8 h.

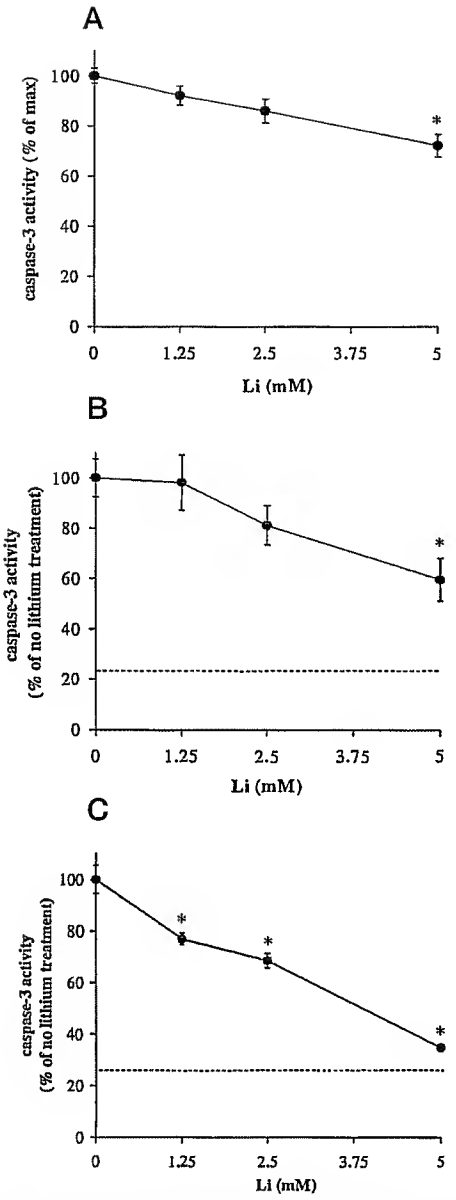

FIG. 4. **Effect of acute and chronic lithium treatment on staurosporine-induced caspase-3 activity.** *A*, caspase-3 activity was measured in untransfected SH-SY5Y cells that were pretreated with 1.25, 2.5, and 5 mM lithium for 24 h and then treated with 0.5 μM staurosporine for 4 h. Values shown are the percent of caspase-3 activity in cells treated with staurosporine alone. Mean ± S.E., $n = 3$ experiments; $^*p < 0.05$ (ANOVA) compared with cells treated with 0.5 μM staurosporine alone. HA-GSK-3β-transfected cells were pretreated with 1.25, 2.5, and 5 mM lithium for 24 h (*B*) or for 7 days (chronic treatment) (*C*) and then treated with 0.1 μM staurosporine for 3 h. Values shown are the percent of caspase-3 activity in HA-GSK-3β-transfected cells treated with 0.1 μM staurosporine alone. *Dotted lines* indicate maximal caspase-3 activity in vector-transfected cells treated with 0.1 μM staurosporine for 3 h without lithium pretreatment. Mean ± S.E., $n = 3$ experiments, $^*p < 0.05$ (ANOVA) compared with HA-GSK-3β transfected cells treated with 0.1 μM staurosporine alone.

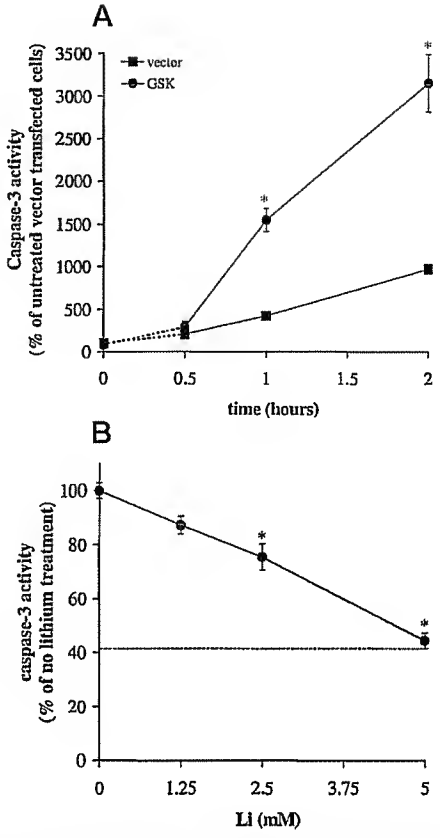

FIG. 5. **Effects of HA-GSK-3β transfection and acute lithium treatment on heat shock-induced caspase-3 activity.** *A*, HA-GSK-3β- and vector-transfected cells were subjected to heat shock (45 °C, 0.5 h) (*dotted line*) and then transferred to a 37 °C chamber for 0.5 to 1.5 h (*solid line*). Values are expressed as the percent of caspase-3 activity in untreated vector-transfected cells. Means ± S.E., $n = 3$ experiments; $^*p < 0.05$ Student's *t* test comparing caspase-3 activity in HA-GSK-3β-transfected cells to vector-transfected cells. *B*, HA-GSK-3β-transfected cells were pretreated with 1.25, 2.5, and 5 mM lithium for 24 h, then subjected to heat shock (45 °C, 0.5 h) and then transferred to a 37 °C chamber for 1 h. Values are expressed as the percent of caspase-3 activity in HA-GSK-3β-transfected cells not pretreated with lithium. *Dotted line* indicates caspase-3 activity in vector-transfected cells not treated with lithium, subjected to heat shock (45 °C, 0.5 h), then transferred to a 37 °C chamber for 1 h. Means ± S.E., $n = 3$ experiments; $^*p < 0.05$ (ANOVA) comparing caspase-3 activity in lithium-pretreated cells to those not pretreated with lithium.

within a few hours of treatment (40), effects also observed in this investigation. Furthermore, we observed approximately a linear concentration-dependent activation of caspase-3 within the range of 0.1 to 1 μM staurosporine. In cells overexpressing GSK-3β, the staurosporine concentration-dependent activation of caspase-3 and of caspase-9 was shifted to the left, so greater activation of these caspases were achieved after treatment with low concentrations of staurosporine. These findings indicated that GSK-3β facilitated staurosporine-induced apoptosis. This conclusion was further confirmed by the findings of greater PARP proteolysis and morphological changes denoting apoptotic cells in GSK-3β-transfected cells than control cells after exposure to staurosporine. Furthermore, the GSK-3β inhibitor, lithium, attenuated the facilitation of staurosporine-induced caspase-3 activity in cells overexpressing GSK-3β. Taken together, these results demonstrate that GSK-3β is a facilitator of the apoptosis-signaling cascade induced by staurosporine.

Our results extend those of Pap and Cooper (23), who recently presented evidence that GSK-3β is proapoptotic. In their studies, transient transfection of GSK-3β in PC12 cells and Rat-1 fibroblasts caused 60 to 70% of cells to spontaneously undergo apoptosis with no additional treatment within 48 h in a caspase-3-dependent manner. Our results differ from those of Pap and Cooper (23) in that a 3.5-fold overexpression of

GSK-3β alone failed to alter caspase-3 activity or cell survival, a difference that may be due to differential cell susceptibilities to the action of GSK-3β or to differences in the expression levels of GSK-3β, which were not reported by Pap and Cooper (23). Regardless of this difference, both studies clearly support the conclusion that GSK-3β is an important modulator of cell survival.

In addition to staurosporine-induced apoptosis, overexpression of GSK-3β potentiated, and lithium treatment attenuated, caspase-3 activation following subjection of cells to heat shock. A variety of strategic responses are initiated in cells to limit the deleterious consequences of stressors, as modeled by heat shock, such as alterations in the activities of signaling protein kinases and induction of the expression of heat shock proteins (see Ref. 47 for review). In SH-SY5Y cells as well as many other types of cells, the responses to a 30-min period of heat shock (45 °C) are adequate to support survival; thus, little caspase-3 activation was observed following heat shock in control cells in this study. Both activation of phosphatidylinositol 3-kinase (48) and inactivation of GSK-3β (49) have been reported to follow heat shock, responses that may contribute, along with others, to protection from heat shock. The results of the present study suggest that adequate inactivation of GSK-3β is critical for cell survival, as excessive GSK-3β resulted in a massive activation of caspase-3 following heat shock, whereas inhibition of GSK-3β by lithium in cells overexpressing HA-GSK-3β protected cells from heat shock-induced caspase-3 activation.

The results presented here suggest two complementary conclusions, that activation of GSK-3β facilitates apoptosis, and that the anti-apoptotic actions of agents that stimulate the phosphatidylinositol 3-kinase/Akt pathway (17) may be due in part to the inhibitory effect of phosphatidylinositol 3-kinase/Akt signaling on GSK-3β activity. The mechanism by which decreased GSK-3β activity contributes to neuroprotection and increased GSK-3β activity contributes to apoptosis remains unknown. One possible mechanism is the regulation by GSK-3β of β-catenin. Increased GSK-3β activity facilitates degradation of β-catenin (50), and reduced β-catenin and the associated reduction in the activity of Tcf/Lef transcription factors has been linked to decreased cell survival (51–53). Additionally, we have found that activation of the heat shock factor-1 transcription factor and the associated expression of heat shock protein-70, which is known to protect against cell death (54, 55), were impaired by expression of GSK-3β and restored by lithium treatment (56). However, since GSK-3β affects a large number of signaling systems, further investigation is necessary to identify those that are critical for its facilitatory action on apoptosis.

Regardless of the mechanism by which GSK-3β facilitates apoptosis, it is evident that inhibition of GSK-3β by lithium reduced caspase-3 activation after both staurosporine and heat shock treatments of GSK-3β-transfected cells. Although lithium has been reported to affect a variety of other targets (26) that cannot completely be discounted as contributory, the accentuated protective effects of lithium in GSK-3β-transfected cells suggests that inhibition of GSK-3β accounts for the protection from apoptosis conferred by lithium. During the last few years several studies (reviewed in Ref. 26), especially those from Chuang and co-workers, have shown that lithium protects neurons from the deleterious effects of a wide variety of insults, such as ischemia (30) and activation of excitatory amino acid receptors (27, 57). The findings in this study raise the possibility that these neuroprotective actions of lithium may occur at least in part because of its capacity to inhibit GSK-3β.

In summary, the results reported here demonstrate for the first time that modest increases in GSK-3β facilitate apoptosis in two model systems, including apoptosis induced by staurosporine and by heat shock. Furthermore, the inhibitory effect of lithium on GSK-3β and its attenuation of GSK-3β-facilitated apoptosis suggest that some of the widely reported neuroprotective effects of lithium may result from this action.

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